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TGFB 1, PTGS 2 Genes Expression during Dynamics of Wound Healing and with the Treatment of Melanin

AS Dranitsina[1]*, OV Taburets[2], KO Dvorshchenko[1], DM Grebinyk[2], TV Beregova[3] and LI Ostapchenko[2]

[1]Research Laboratory of Biochemistry, "Institute of Biology", Taras Shevchenko National University of Kyiv. Kyiv, Ukraine.
[2]Chair of Biochemistry, "Institute of Biology", Taras Shevchenko National University of Kyiv. Kyiv, Ukraine.
[3]Research Laboratory of Pharmacology and Experimental Pathology, Educational and Scientific Center "Institute of Biology", Taras Shevchenko National University of Kyiv. Kyiv, Ukraine.

ABSTRACT

Wound repair in adult skin begins with an acute inflammatory phase and ends with the formation of a permanent scar. TGF-β_1 and COX2 are factors identified as reduced in scarless healing. Melanin, which is produced by Antarctic black yeast-like fungi *Nadsoniella nigra*, strain X1-M, has expressed cytoprotective effect and can be offered as a new dermotropic drug. Study was conducted on rat model of full-thickness skin wound and purulent necrotic wound. In each model one group was a control, while in others wound healing occurred without drugs or with administration of 0,5 % carbopol or with both 0,5 % carbopol and 0,1% melanin. Level of *Tgfb1*, *Ptgs2* genes mRNA was determined with quantitative RT-PCR. Significant elevation of *Tgfb1* and *Ptgs2* genes expression was observed during healing of full-thickness skin wound and purulent necrotic wound. Reduction of expression of these genes on the background of absence of scarring was observed upon administration of melanin. Obtained results may indicate the advisability of melanin for the treatment of inflammatory processes.

Keywords: full-thickness skin wound, purulent necrotic wound, Ptgs2, Tgfb1 gene expression, melanin

Corresponding author

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INTRODUCTION

Wound healing is an inherent process, which restores the integrity of skin as quickly as possible. Restoration of the skin is essential, due to the skins importance in survival through the prevention of infection, fluid loss and other vital functions [1]. Wound repair in adult skin begins with an acute inflammatory phase and ends with the formation of a permanent scar. In contrast, early gestation fetal wounds (first and second trimester) heal in a near perfect fashion, rapidly and without the production of a scar [1, 2]. Often there are also complications of scar-degenerative origin in the area of wound - hypertrophic and keloid scars; scar restrictions (contractions) of movements scar hernia and diastase on the basis of muscle atrophy, sores and rarely - tumors [1-3].

Identification of differences in above mentioned two types of healing could identify factors that promote scar tissue generation. This correlation between factors identified as reduced in scarless healing and the inhibition of expression of those factors in adult wounds to reduce scarring has been especially true for transforming growth factor β (TGF-β_1) [1, 2, 4, 5].

This cytokine causes multiple effects on different types of cells participating in regulation of cell growth, differentiation, apoptosis, immune response and the remodeling of the extracellular matrix, particularly through the intracellular SMAD pathway, CTGF (CCN2) - a downstream mediator of TGF-β1; the proteoglycan decorin and the binding protein p311 etc. [1, 5-7]. TGF-β_1 was shown to promote scar tissue deposition when introduced into scarless wounds [2, 4, 8, 9]; As a result of these findings and others implicating TGF-β_1 in fibrosis, the effect of down-regulating this molecule was tested in adult skin and found to reduce scar formation [1 - 4, 8, 9].

A key feature of scarless fetal healing appears to be a lack of inflammation in response to the wounding event, with a decrease in anti-inflammatory interleukin - IL-10 against the backdrop of the release of proinflammatory cytokines, including IL-2, IL-6, IL-8, tumor necrosis factor α (TNFα), etc., as well as other inflammatory mediators [2, 8, 10].

Metabolites and enzymes of the arachidonic acid cascade, including the cyclooxygenase-2 - COX-2 - enzyme (encoded by *Ptgs2* gene), and its enzymatic product prostaglandin E_2 (PGE_2), are known to be critical mediators of the inflammatory response. *Ptgs2* undergoes immediate-early up-regulation in response to an inflammatory stimulus, such as a wound and a burn [2, 11]. In turn, prostaglandins regulate the induction of vascular permeability, infiltration and activation of inflammatory cells, synthesis of molecules of cell adhesion and migration of neutrophils to damaged tissue. Furthermore, PGE_2, a COX-2 product, shown to mediate many processes in the skin, caused a delay in healing and the production of a scar when introduced into early fetal wounds [2, 11, 12].

The most common in clinical practice, there are some treatments for open wounds and superficial skin burns II-IIIA degree that does not require surgery, the use of ointments, gels, bandages, antibiotics. However, the high cost of treatment and lack of effectiveness of existing drugs, particularly in the treatment of hypertrophic scarring encourage researchers to seek new methods of treatment [1, 13, 14]. At the same time, understanding the mechanisms underlying in scarring, particularly hypertrophic, help in the treatment aimed to prevent or reduce scarring [1, 2].

Melanin - pigment of skin, hair, iris, substantia nigra of the brain and so on - belongs to the polyphenol compounds. It is known that these compounds exhibit reparative, antioxidant, anti-inflammatory, wound healing, immunomodulatory and antitumor properties [15-17].

We have previously shown that melanin, which is produced by Antarctic black yeast-like fungi *Nadsoniella nigra*, strain X1-M, from Galindez island's vertical cliffs (Ukrainian Antarctic Station "Akademik Vernadsky") has expressed cytoprotective effect, promotes rapid healing of wounds of various etiology and can be offered as a new dermotropic drug [18, 19].

Considering the above, the aim of this research was to assess *Tgfb1*, *Ptgs2* genes expression during dynamics of wound healing and with the treatment of melanin.

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MATERIALS AND METHODS

Positive findings of ethical expertise were adopted on session of Bioethical Commission of ESC "Institute of Biology" of Taras Shevchenko National University of Kyiv on 26 June 2013. All animals were handled humanly according to rules outlined in "Guide for the Care and Use of Laboratory Animals" (2011), and Order of Ukraine №3447-IV"About defense of animals from abusive handling" from 21 February 2006.

Animal model. All experiments were carried out on white non-strain female rats weighing 200 - 250 g, n = 96, which were divided into two groups, each group was divided into four subgroups. Before the experiment, the rats were kept in quarantine and were marked by given them notches on ears. In each model animals without experimental skin wounds were used as a control (first group). Before the experiment epilation was performed in the shoulder-blade area. When animas were injured they were anesthetized by sodium thiopental ("BiochemieGmbH", Austria), at a dosage of 50 mg / kg and healing occurred without drugs (second group). Wounds of rats of third group were treated only with 0,5 % carbopol (universal solvent drugs to make them gel-like consistency, "Carbopol 980"). Animals of fourth group got 0,1% melanin (produced by Antarctic black yeast-like fungi *Nadsoniella nigra*, strain X1-M,and received by us microbiologically) dissolved in 0,5% carbopol for wounds' healing.

Skin wound model

Model of full-thickness skin wound: Plate wounds were reproduced on epilated skin in anesthetized rats. To do it, skin was cut using surgical scalpel and forceps, 1 × 1 sm^2. Treatment begun immediately after wounds reproduction until healing [20].

Burns caused by calcium salt solution: Chemical skin burns were caused by the introduction of 0,1 ml $CaCl_2$. The attention was payed to the standardization of wounds received, the size of which was not to exceed 400 mm^2. On 4-5 days necrotomy of the affected area was performed and then treatment of wounds begun until healing [21].

Quantitative RT-PCR: RNA was isolated following Chomczynski and Sacchi [22]. Conditions of cDNA synthesis and quantitative Real-time PCR (qRT-PCR) were according to instruction of the SYBR Green I kit "Thermo Scientific Verso SYBR Green 1-Step qRT-PCR ROX Mix" ("Thermo Scientific", Lithuania): cDNA synthesis 50°C – 30 min; initial denaturation 95°C – 15 min; following 40 cycles: denaturation ДНК 95°C – 15 s; annealing 50ºC – 35 s; extension 72° C – 30 s; final extension 72°C – 5 min. Primers were designed into exon-intron junctions to avoid amplification of genomic DNA. The primers used were (designed using Primer-BLAST): for *Tgfb1* – forward - CTTCAGCTCCACAGAGAAGAACTGC and reverse - CACGATCATGTTGGACAACTGCTCC; for *Ptgs2*: forward – TGCTGTTCCAACCCATGTCA, reverse – TGTCAGAAACTCAGGCGTAGT; for *Actb* (as an endogenous control gene) – forward – TGGGACGATATGGAGAAGAT and reverse – ATTGCCGATAGTGATGACCT. We run at least 3 replicates for each gene, RNA sample and every primer. The melting curve analysis was carried out to assess whether the intercalating dye in qRT-PCR assays produced single, specific products without formation of primer dimers.

The relative quantification was calculated by the comparative C_T method: the data were analyzed using the equation $2^{-\Delta\Delta CT}$, where ΔΔCT = [C_T of target gene – C_T of housekeeping gene]treated group – [C_T of target gene – C_T of housekeeping gene]untreated control group. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression, normalized to the housekeeping gene (*Actb*), and relative to the untreated control. In a validation of ΔΔCT calculation the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal (Ex =($10^{-1/slope}$)-1, the absolute value of the slope of ΔCT vs. log input is < 0,1.

Statistics: Statistical processing of experimental results was carried out in "GraphPad Prism 5" ("GraphPad Software Inc.", USA). Type of data distribution in groups was checked with Shapiro-Wilk test. As data were distributed normally ($p > 0,05$), two-way ANOVA was conducted to determine the significance of difference between means with Bonferroni post test. Difference between means was judged as statistically significant if $p \leq 0,05$. Mean and standard deviation (SD) were calculated for each group.

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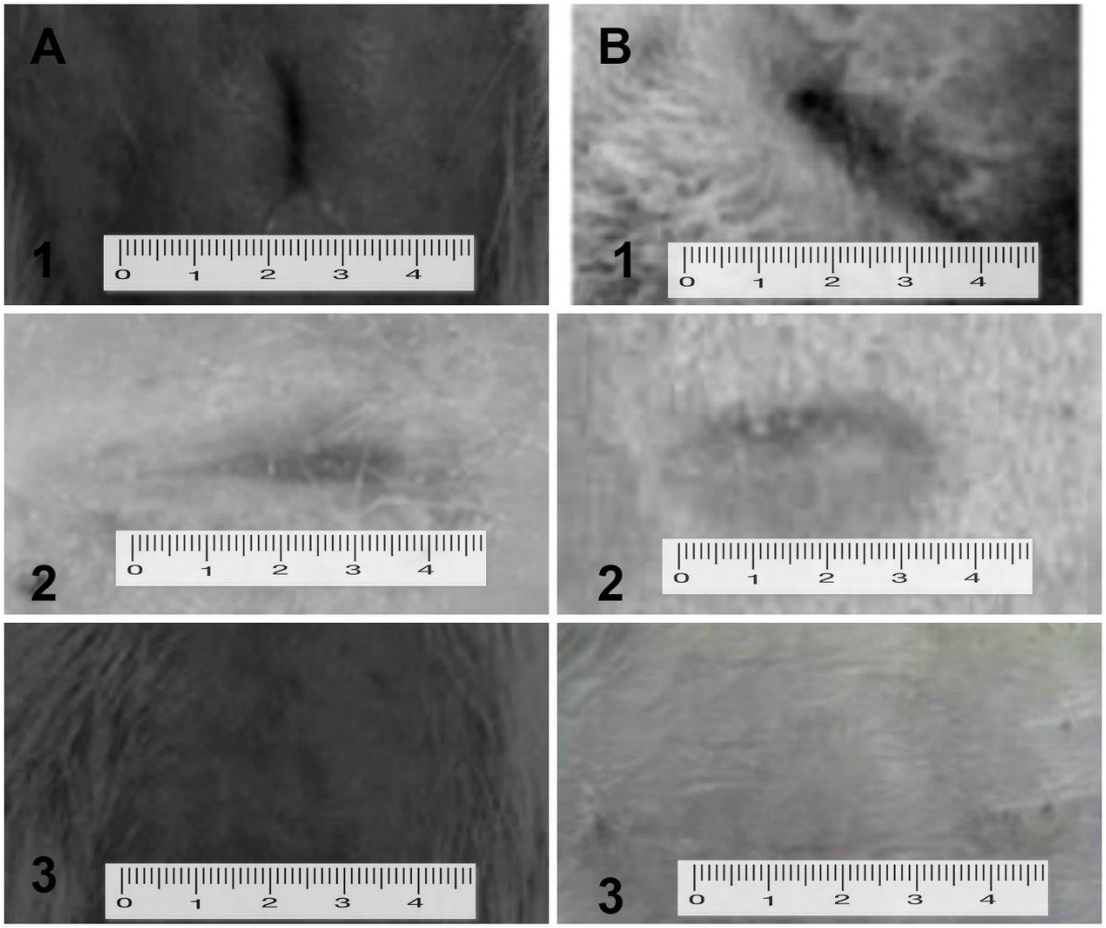
RESULTS AND DISCUSSION

We showed the absence of scarring after complete wound closure both in the model of full-thickness skin wound and in the model of the chemical burns caused by subcutaneous injection of calcium salt, which led to extensive tissue necrosis, with administration of melanin in contrast to the healing wound surfaces without treatment (Figure 1).

Figure 1: The surface of a) full-thickness and b) purulent necrotic wound after complete wound closure 1 – wound; 2 – wound + 0,5 % carbopol; 3 – wound + 0,1 % melanin dissolved in 0.5% carbopol.



Also, our previous studies found that rapid wounds healing of various etiologies using melaninin occurred in the initial phase of the regeneration of the skin by analysis of active contraction of the wound surface in dynamics on 3, 5, 7, 9, 14, 21 and on 30-th days. Besides this, melanin revealed a strong bactericidal effect on *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans*, that made the appropriate application of the drug for the treatment of infectious inflammatory processes [19].

We found, that the level of *Tgfb1* gene expression in rats with full-thickness wounds was 5,2, 6,7, 5 and 3 times ($p \leq 0{,}0001$) higher of the control value on 3, 6, 9 and 14 day respectively (Figure 2). The levels of this gene mRNA in the second and the third group of animals were not significantly different.

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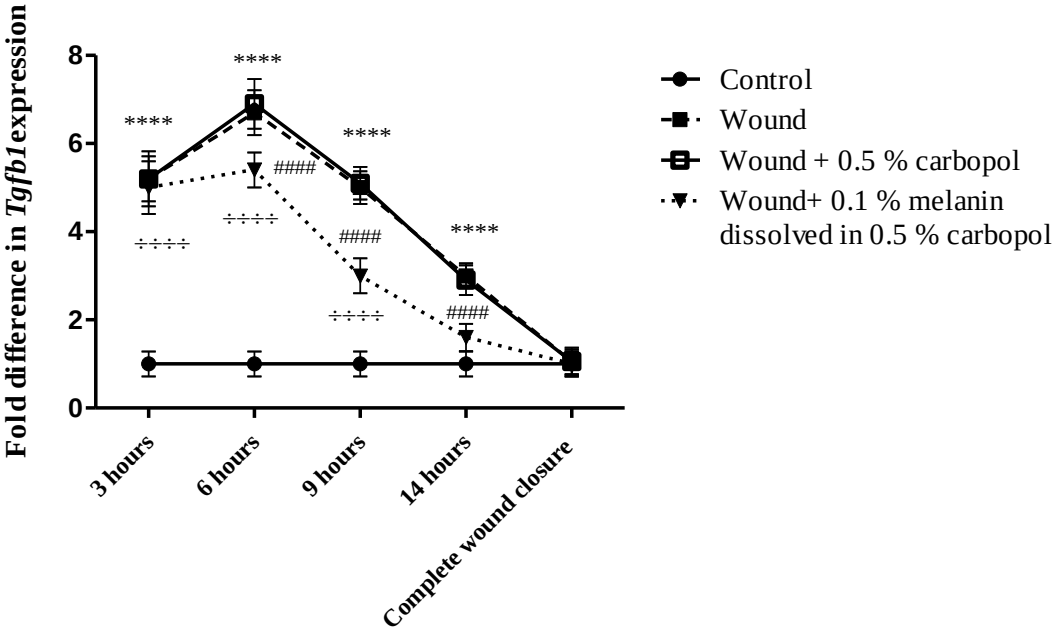
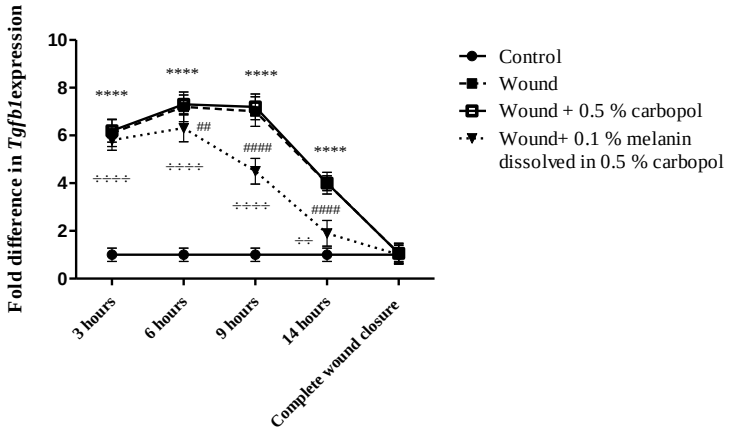
Figure 2: *Tgfb1* gene expression during dynamics of full-thickness wound healing and with the treatment of melanin. ** – p ≤ 0,0001 wounds in relation to control; #### - p ≤ 0,0001 wounds treated with melanin compared to the animals with wounds; ÷÷÷÷ - p ≤ 0,0001 wounds treated with melanin in relation to control.**



However, in rats which wounds were treated with melanin the level of *Tgfb1* gene expression was 1,2, 1,7 and 1,9 times (p ≤ 0,0001) lower than in group of untreated injury on 6, 9, 14 day accordingly, and less elevated than the control values: 5, 5,4 and 3 times (p ≤ 0,0001) on 3, 6 and 9 day respectively. On 14 day this index didn't differ from control value. The level of *Tgfb1* mRNA was at the control values in the second, third and fourth groups of animals in the complete closure of the wound. In particular, we have previously shown, that complete full-thickness wound closure in the group of rats without treating occurred on the 23,2 ± 1,0 day, in group of animals with melanin - on 21,0 ± 0,5 day [19].

We found higher values of *Tgfb1* gene expression in purulent necrotic wounds in comparison with full-thickness ones. Thus, in the group of animals with untreated lesions this gene expression was 6,1, 7,2, 7 and 4 times (p ≤ 0,0001) higher of the control value on 3, 6, 9 and 14 days respectively (Figure 3).

Figure 3: *Tgfb1* gene expression during dynamics of purulent necrotic wound healing and with the treatment of melanin. ** – p ≤ 0,0001 wounds in relation to control; #### - p ≤ 0,0001, ## - p ≤ 0,01 wounds treated with melanin compared to the animals with wounds; ÷÷÷÷ - p ≤ 0,0001, ÷÷ -, p ≤ 0,01 wounds treated with melanin in relation to control**



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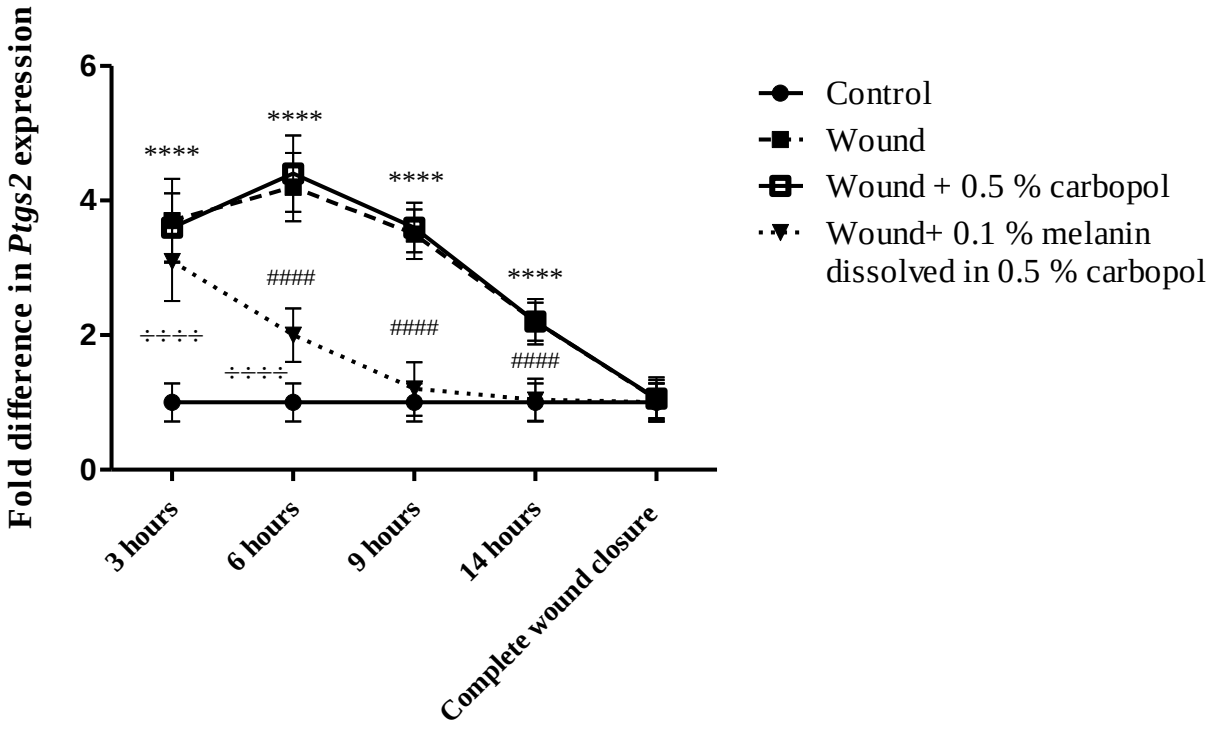
The levels of this gene mRNA in the second and the third group of animals were not significantly different. However, in rats which wounds were treated with melanin, the level of *Tgfb1* gene expression was 1,1, ($p \leq 0,01$), 1,6 and 2,1 times ($p \leq 0,0001$) lower than in the second group on 6, 9, 14 day accordingly, and less elevated than the control values: 5,8, 6,3, 4,5 ($p \leq 0,0001$) and 1,9 times ($p \leq 0,01$) on 3, 6, 9 and 14 day respectively. The level of *Tgfb1* mRNA was at the control values in the second, third and fourth groups of animals in the complete closure of the wound. Besides this, we have previously shown, that complete purulent necrotic wound closure in the group of rats without treating occurred on the 38,1 ± 0,5 day, in group of animals with administration of melanin - on 36,0 ± 0,7 day [19].

As a result of further experimental studies showed, the level of *Ptgs2* gene expression in rats with full-thickness wounds was 3,7, 4,2, 3,5 and 2,2 times ($p \leq 0,0001$) higher of the control value on 3, 6, 9 and 14 добу day respectively (Figure 4). The levels of this gene mRNA in the second and the third group of animals were not significantly different.

Figure 4: *Ptgs2* gene expression during dynamics of full-thickness wound healing and with the treatment of melanin. ** – p ≤ 0,0001 wounds in relation to control; #### - p ≤ 0,0001 wounds treated with melanin compared to the animals with wounds; ÷÷÷÷ - p ≤ 0,0001 wounds treated with melanin in relation to control.**



However, in rats which wounds were treated with melanin this index was 2,1, 2,9 and 2,1 times ($p \leq 0,0001$) lower than in group of untreated injury on 6, 9 i 14 day accordingly, and less elevated than the control values: 3,1 and 2 times ($p \leq 0,0001$) on 3 and 6 day respectively. On 9 day the level of *Ptgs2* expression didn't differ from control value. The level of *Ptgs2* mRNA was at the control values in the second, third and fourth groups of animals in the complete closure of the wound.

We found higher values of *Ptgs2* gene expression in purulent necrotic wounds in comparison with full-thickness ones. Thus, in the group of animals with untreated lesions this gene expression was 4, 4,5, 4,6 and 2,8 times ($p \leq 0,0001$) higher of the control value on 3, 6, 9 and 14 days respectively (Figure 5).

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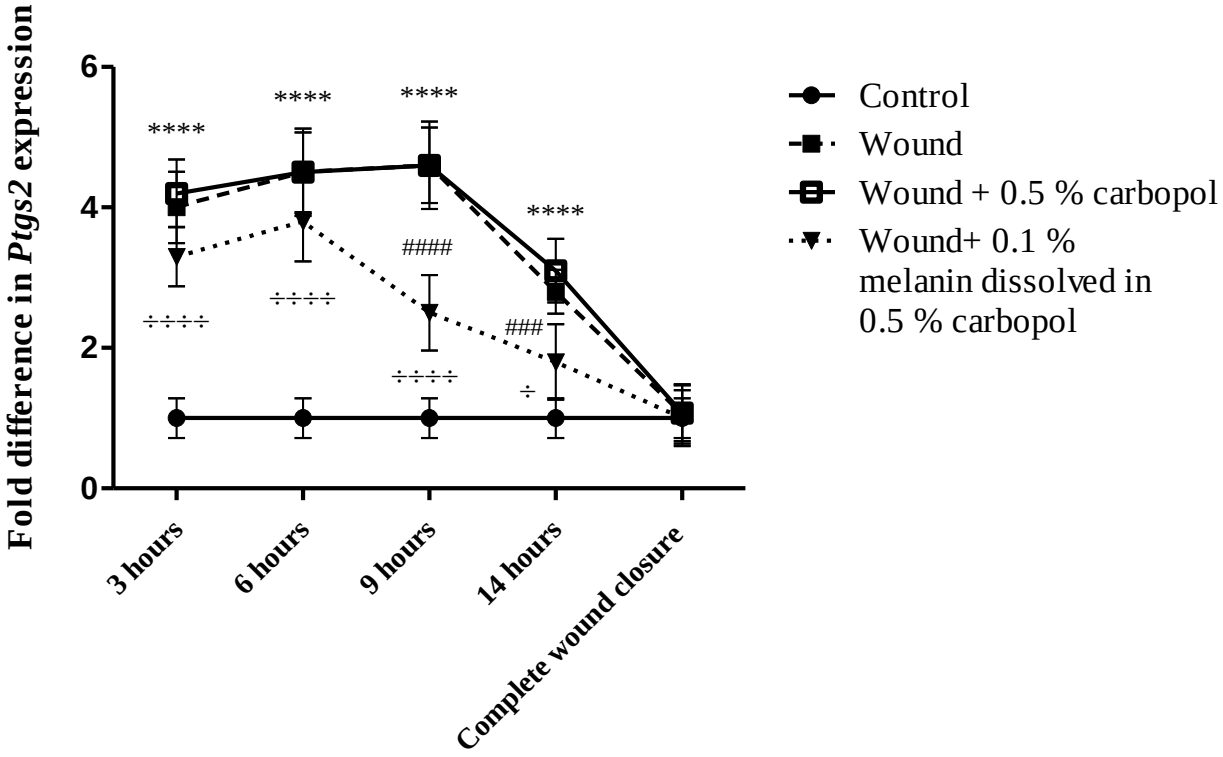
Figure 5: *Ptgs2* gene expression during dynamics of purulent necrotic wound healing and with the treatment of melanin. ** – p ≤ 0,0001 wounds in relation to control; #### - p ≤ 0,0001, ### - p ≤ 0,001 wounds treated with melanin compared to the animals with wounds; ÷÷÷÷ - p ≤ 0,0001, ÷ -, p ≤ 0,05 wounds treated with melanin in relation to control**



The levels of this gene mRNA in the second and the third group of animals were not significantly different as in the previous model of skin damage. In rats which wounds were treated with melanin this index was 1,8, (p ≤ 0,0001) and 2,1 1,6 (p ≤ 0,001) times lower than in group of untreated injury on 9 and 14 day accordingly, and less elevated than the control values: 3,3, 3,8, 2,5 (p ≤ 0,0001) and 1,8 (p ≤ 0,05) times on 3, 6, 9 and 14 day respectively. The level of *Ptgs2* mRNA was at the control values in the second, third and fourth groups of animals in the complete closure of the wound.

Wound healing is a dynamic process with at one end of the spectrum an over exuberance resulting in pathological scarring while at the other end a non-healing or chronic wound. Hypertrophic scarring has been shown to be more common following certain injuries such as burns, delayed epithelization or wounds occurring in areas of high tension for example the deltoid and sternal regions or areas of movement. Unlike keloids, hypertrophic scars remain within the boundary of the original injury and hypertrophic scars can regress with time [1, 13, 14, 23].

As noted, it has been known since the 1970's that scarring is not required for wound healing with early human gestational fetuses healing cutaneous wounds perfectly without the formation of scar tissue [1, 2]. At the same time, as it turned out, a key feature of embryonic wound healing without scarring is a lack of inflammation in response to injury [1, 2, 13, 24]. Thus, established in our study increase in *Tgfb1* gene mRNA in rats with full-thickness wounds and purulent necrotic wound reflects both the inflammation and, consequently, the formation of scars, which we were found after healing of untreated wounds.

Because, potential sources of melanocytes are significantly damaged in full-thickness wounds, while in purulent necrotic wounds powerful inflammatory response also leads to pigmentation disorders and scarring [2, 25]. In addition, the above mentioned gene in wounds not only stimulates angiogenesis, proliferation of fibroblasts, collagen synthesis and new extracellular matrix remodeling, but also the

development of inflammation [1, 2, 9]. Not only have hypertrophic scar tissue shown more *Tgfb1* expression, but they have also been shown to have a prolonged expression of the TGF-β receptors (TβRI for TGF-β_1) compared to normal skin. Studies have also indicated increased expression and phosphorylation of the receptor Smads-2 and/or 3 in hypertrophic scarring [1, 5, 26].

We found no scarring during healing of both full-thickness skin wound and burns caused by calcium salt solution with administration of melanin. Upon the treatment of melanin we observed reduction of *Tgfb1* expression, which is consistent with other work, in which *Tgfb1* inhibition led to decrease of inflammation and wound healing was also with a lower degree of scarring [1, 2, 6, 9].

Besides increase in *Tgfb1* expression, other putative mechanisms which may underlie the pathogenesis of hypertrophic scars include excessive inflammation, excessive angiogenesis, altered levels of matrix metalloproteinases, growth factors, and delayed apoptosis of fibrotic myofibroblasts either due to p53 genetic alterations or tensile forces across the wound [1, 2].

Thus, we found elevation of *Ptgs2* gene expression in rats with full-thickness wounds and purulent necrotic wound, that also indicated the inflammation, which was confirmed by the formation of scars in rats without treatment and was consistent with other studies [1, 2, 9]. Furthermore, *Ptgs2* gene as its product, PGE_2, shown to mediate many processes in the skin, caused a delay in healing and the production of a scar when introduced into early fetal wounds.

Thus, it was shown, that the introduction of PGE_2 induced inflammation in fetal wounds by further activation of inflammatory cells and stimulated scarring by increasing fibroblast proliferation and collagen synthesis [2, 11, 12]. High COX-2 expression and PGE_2 production are believed to contribute to the uncontrolled proliferation of tumor cells, as well as to mobilization and invasion, so it is conceivable that higher COX-2 expression in fibroblasts could stimulate their own activation, migration, and/or proliferation, augmenting scar tissue production [2]. At the same time *Ptgs2* and PGE_2 treatment could be both delaying healing and promoting scar tissue deposition through increases in the pro-fibrotic TGF-β [2].

Upon the treatment of melanin we observed reduction of *Ptgs2* expression as for *Tgfb1*, which is consistent with other work, in which *Tgfb1* inhibition led to decrease of inflammatory process and wound healing was also with a lower degree of scarring [2 , 11, 12].

To date, there remains no definitive treatment to either prevent or reduce any form of scarring. Clinical studies on current treatments are often inadequate due to small numbers of patients, lack both of well-designed controls and standardization in scar outcome measurements, for example at the expense of lack definitive animal model available. If an effective treatment for hypertrophic scars following burns injury is to be developed then further work must be carried out to understand the basic mechanisms of pathological scarring [1, 2, 13, 14].

A number of groups are studying the manipulation of TGF-β in the prevention of both normal scarring and hypertrophic scarring [1, 27]. TGF-β neutralizing antibodies have been shown to inhibit fibrosis in a number of animal models, however, reduction in TGF-β signaling has been linked with chronic or non-healing wounds [1, 28]. Blocking TGF-βthrough a number of natural TGF-βinhibitors, such as decorin, biglycan, LAP, may block the fibrotic TGF-βresponse, but not affect the TGF-βimmune response [1, 29]. Other methods of manipulating TGF-β include blocking the TGF-βreceptors with kinase inhibitors (for example SD-208), but few clinical studies have been performed on the manipulation of TGF-β_1 and its isoforms to prevent dermal scarring [1]. Search for agents to inhibit a TGF-β_1 and a COX-2 - mediated inflammatory pathway to reduce inflammation and scar formation further continues [1, 2, 13, 14].

Thus, found changes in *Tgfb1* and *Ptgs2* gene expression during dynamics of full-thickness wounds and purulent necrotic wound healing indicated development of pathological processes, in particular inflammation, which was confirmed by the formation of scars in rats without treatment of melanin.

Therefore, we can make assumptions about the impact of melanin to reduction of these expression, and thereafter the synthesis of prostaglandin E_2 and TGF-β_1, which is a prerequisite for rapid wound healing

without expressed scarring [1, 2, 9, 12].

Other authors have found the opposite effect of melanin in cell culture: by means of various factors melanocytes stimulated proliferation of fibroblasts, increased the synthesis of collagen and extracellular matrix and vice versa - activated TGF-β_1 signaling pathway, and therefore contributed to the development of pathological scarring [30]. But in our study for the first time we showed not only lower expression levels of *Tgfb1* and *Ptgs2* during dynamics of full-thickness wounds and purulent necrotic wound healing, but no scarring with administration of melanin, which was consistent with its proven cytoprotective effect on the gastric mucosa, etc. [15, 18, 19].

As noted, melanin protects the body from ultraviolet and X-rays, has radioprotective, stress protective, cytoprotective, antioxidant and anti-inflammatory action [15-19]. It is able to bind to collagen fibers, stabilizes them and prevents the formation of unwanted cross-links between the amino acids [15].

Melanin's the main sources, involved in this study, are microorganisms that live in extreme conditions, use melanin to protect against harmful UV radiation, and transform energy into a safe amount of heat. Due to its properties melanin absorbs up to 99.9% of UV light and prevents the formation of free radicals at a minimal level and may be stronger radioprotectors and antioxidant compared to other melanin [16, 19].

Regarding the possible mechanisms of influence of melanin as polyphenolic compounds on analyzed gene expression during healing of skin lesions of various etiologies, above all, it should be noted its pronounced cytoprotective effect. It reduces the activity of lipid peroxidation, increases the activity of enzymes of antioxidant system, preventing DNA damage; impact on production of cytokines: TNF-α, IL-6, VEGF, etc. by, for example, the influence on the expression of nuclear receptors PPAR [31]; increases eNOS expression and secretion of anti-inflammatory cytokines to reduce the intensity of inflammation and scarring during healing [15-19]. Further study should be performed to elucidate the specific molecular mechanisms of melanin effect on wound healing.

CONCLUSION

Current study demonstrates elevation of *Tgfb1* and *Ptgs2* genes expression level during healing of full-thickness skin wound and purulent necrotic wound. Reduction of expression of these genes on the background of absence of scarring was observed upon administration of melanin. Obtained results may indicate the advisability of applying drug for the treatment of inflammatory processes.

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